Salidiuretic Action by Genistein in the Isolated, Perfused Rat Kidney

Ignacio Giménez, Rosa M. Martinez, Manuel Lou, José A. Mayoral, Ricardo P. Garay, José O. Alda

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(Hypertension. 1998;31:706-711.)

Key Words: genistein • isoflavonoids • kidney • natriuresis • rats

Rat urine contains compounds potently inhibiting furosemide-sensitive Na-K-Cl cotransporters (cotransport inhibitory factors [CIF]).1-4 Recently, one of such compounds was purified and structurally characterized as the isoflavonoid phytoestrogen equol (3,4-dihydro-3-(4-hydroxyphenyl)-2H-1-benzopyran-7-ol, see structure in Fig 1; see also Reference 2). Equol has similar potency as furosemide to inhibit cotransport, is a modest natriuretic agent in the rat, and can be excreted at urinary concentrations sufficient as furosemide to inhibit cotransport, is a modest natriuretic agent in the rat, and can be excreted at urinary concentrations sufficient

Measurement of Na–K–Cl Cotransport Fluxes in LLC-PK1 Cells

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At the end of the incubation period, cells were washed three times with cold MgCl2 110 mmol/L and lysed with 30 μL of ethanol. Rubidium contents were measured by atomic absorption spectrophotometry in an ATU Unicam 929 (Unicam Ltd). Protein contents were measured by the use of a BIO-RAD protein assay (BIO-RAD Labs, Gmbh). Rubidium uptake was calculated as rubidium content per milligram of protein divided by the incubation time. To calculate Na–K–Cl cotransport activity, rubidium uptake in the presence of bumetanide was subtracted from that in its absence.

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access to water. The animals then were anesthetized with sodium thiopental (50 to 60 mg/kg), the abdominal cavity was exposed, and the right ureter was cannulated with PE-10 tubing. The right renal artery was cannulated through the superior mesenteric artery. The perfusion was initiated via a rotary motion of 60 mL of Krebs-Henseleit bicarbonate buffer medium containing 75 g/L fraction V bovine albumin (Miles Inc), 1 g/L glucose, 0.5 g/L creatinine, and a mixture of amino acids (in mmol/L): L-methionine 0.5, L-alanine 2, glycine 2, L-serine 2, L-proline 2, L-isoleucine 1, L-arginine 1, and L-aspartic acid 3. The perfusion solution was saturated with 95% O2/5% CO2, maintained at 37°C, and filtered (8.0 μm).

A 20- to 30-minute stabilization period was allowed before any measurements were taken. During this period, renal perfusate flow was adjusted to obtain a steady effective perfusion pressure in the range 95 to 100 mm Hg. Renal vascular resistance (RVR) was calculated as the ratio between effective perfusion pressure and renal perfusate flow (for details see Reference 8).

Before the experiment, two (basal) urinary samples were collected directly from the ureteral cannula in preweighed Eppendorf tubes during two consecutive periods of 10 minutes (perfusate samples were withdrawn at the midpoint of each period). Cumulative doses of genistein or furosemide then were administered in the perfusate during four further consecutive periods of 10 minutes and compared with kidneys receiving vehicle.

Urinary (and perfusate) volume, Na, K, and creatinine were measured during each of the above renal periods. Creatinine determinations were used to estimate glomerular filtration rate (GFR). Creatinine was measured by use of a colorimetric assay (BioSystems SA). Na and K were measured in an ATI Unicam 929 atomic absorption spectrophotometer (Unicam Ltd). In some experiments, urinary genistein contents were measured by high-performance liquid chromatography (HPLC).

**Salidiuretic Studies in Rats**

Fourteen rats were placed in metabolic cages 1 week before the study. A control period of 24 hours was allowed to measure basal diuresis, natriuresis, kaliuresis, and creatinine. A group of 7 rats then received a subcutaneous injection of 15 mg/kg genistein (genistein was dissolved in a small amount of DMSO and suspended in 1 mL of 51 mmol/L methanol), and a second group of 7 rats received the same volume of vehicle alone. Urine was collected during the first 6 hours, the 18 following hours, and one further 24-hour period. Urinary sodium and potassium contents were measured in an ATI Unicam 929 atomic absorption spectrophotometer (Unicam Ltd).

**Chemicals**

Daidzein was prepared by two of the investigators (J.A.M. and R.M.M.) from resorcinol by using a previously described method.6 Purity, assessed by HPLC, nuclear magnetic resonance, and mass spectrum was >0.99. All other chemicals were from SIGMA. To test their in vitro or in vivo effects, drugs were weighed and dissolved in water, saline, or DMSO the day of the experiment and directly administered.

**Statistical Analysis**

Values are expressed as mean±SEM. Statistical differences between mean values were determined by use of the nonpaired Student’s t test. Multiple measurement comparison was performed by use of an ANOVA program followed by a nonpaired Student’s t test with Bonferroni correction. Statistical significance was accepted for values of P<.05. IC50 and EC50 values were obtained by linear regression analysis applied to responses between 20% and 80% of the maxima.

**Results**

**Inhibition of Na-K-Cl Cotransport Fluxes by Genistein and Daidzein**

Fig 2 and Table 1 show the effect of genistein and daidzein on Na-K-Cl cotransport fluxes in the LLC-PK1 renal tubular cell line. It can be seen that genistein inhibited cotransport with 3 to 4 times less potency than furosemide (IC50, 34.7 and 10.3 μmol/L for genistein and furosemide, respectively). Daidzein was a poor inhibitor of cotransport, acting with 14 times less potency than furosemide (IC50, 140 and 10.3 μmol/L for daidzein and furo-

[Image 1: Chemical structures of biologically relevant isoflavonoids. Asterisk indicates an asymmetric carbon atom.]

[Image 2: Inhibition by genistein and daidzein of Na-K-Cl cotransport fluxes in the LLC-PK1 renal tubular cell line. Values are given as mean±SEM. Na-K-Cl cotransport activity was equated to the bumetanide-sensitive rubidium uptake. Genistein but not daidzein inhibited cotransport in a range of concentrations similar to that of furosemide. See Table 1 for IC50 values.]
semide, respectively). On the basis of these results, only genistein was tested in the isolated, perfused rat kidney.

Isolated, Perfused Kidney
Genistein and furosemide were tested in cumulative dose-response curves for salidiuretic activity in the isolated, perfused rat kidney. Fig 3 shows that both compounds induced a very substantial increase in diuresis (Fig 3, top), natriuresis (Fig 3, middle), and kaliuresis (Fig 3, bottom), which was highly significant with respect to IPK receiving vehicle. Maximal salidiuretic action was similar for both compounds (Fig 3), whereas genistein was 3 to 5 times less potent than furosemide in terms of active doses (Table 1).

Genistein and furosemide were tested together at doses approximately twice the diuresis EC50 for each respective compound (308 and 100 μmol/L for genistein and furosemide, respectively). In kidneys being perfused in the presence of furosemide, Table 2 shows that genistein was unable to further stimulate salidiuresis. Table 3 shows that indomethacin (10 μmol/L) reduced by ~40% the salidiuresis values measured in the presence of genistein, although the difference did not reach statistical significance. Such inhibitory action of indomethacin reached statistical significance when the measured values were divided by the respective basal values. Thus genistein increased basal values of (1) diuresis by 3.82 ± 0.17 and 2.73 ± 0.35 (P < .05) in the absence and presence of indomethacin, respectively, and (2) natriuresis by 8.24 ± 1.89 and 4.57 ± 0.65 (P < .05) in the absence and presence of indomethacin, respectively.

Genistein was compared with furosemide in IPK for its effects on GFR and RVR. Fig 4 shows that genistein had no significant effect on GFR but was able to significantly reduce RVR with respect to vehicle IPK. Conversely, furosemide did not affect RVR and induced a slight but not significant stimulation of GFR with respect to vehicle IPK. Table 4 shows experiments in which genistein was added to kidneys being perfused in the presence of furosemide 100 μmol/L. It can be seen that furosemide reduced the decrease in RVR by genistein, although the effect did not reach statistical significance. Finally, Table 5 shows that in the presence of indomethacin (10 μmol/L), genistein modestly and not significantly decreased RVR (note, however, that indomethacin did not significantly reduce the action of genistein on RVR).

Isolated rat kidneys were perfused with genistein (308 μmol/L), and urinary genistein concentrations were determined by HPLC. Genistein was rapidly excreted, reaching stationary concentrations of 12 to 15 μmol/L (30 minutes after perfusion of genistein, urinary genistein reached a concentration of 13.4 ± 1.4 μmol/L, n = 4). The presence of furosemide (100 μmol/L) or indomethacin (10 μmol/L) was without significant effect on urinary genistein concentrations (11.0 ± 2.2 and 17.8 ± 6.9 μmol/L for genistein in the presence of furosemide or indomethacin, respectively).

Salidiuretic Activity in Rats
Fig 5 shows the effect of subcutaneous genistein (15 mg/kg) on salidiuresis during the first 6 hours in rats. It can be seen that genistein induced a statistically significant increase in diuresis and natriuresis with respect to vehicle. Conversely, kaliuresis was unchanged with respect to vehicle (Fig 5). Moreover, genistein did not affect the volume or sodium content of urine samples collected between 6 and 42 hours after administration.

Discussion
Plant isoflavonoids are now receiving considerable attention because of their well-documented action on the genesis and

<p>| TABLE 1. Inhibition of Cotransport Fluxes and Salidiuretic Activity in IPK by Genistein and Daidzein vs Furosemide |
|----------------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Cotransport IC50, μmol/L</th>
<th>Diuresis EC50, μmol/L</th>
<th>Natriuresis EC50, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>34.7 ± 1.3 (4)</td>
<td>154 ± 46 (4)</td>
<td>237 ± 92 (4)</td>
</tr>
<tr>
<td>Furosemide</td>
<td>10.3 ± 1.3 (5)</td>
<td>49 ± 14 (3)</td>
<td>56 ± 20 (3)</td>
</tr>
<tr>
<td>Daidzein</td>
<td>140 ± 12 (4)</td>
<td>nm</td>
<td>nm</td>
</tr>
</tbody>
</table>

IPK indicates isolated, perfused kidney; nm, not measured.
The number of experiments is given in parentheses.
proliferation of cancer cells, making them strong candidates to be natural cancer-protective compounds (for review see Reference 6). Epidemiological studies support this hypothesis, with excretion of the greatest concentration of isoflavonoids found in countries with low cancer incidence. Genistein inhibits tyrosine kinase, and this seems a key property, as suggested by a study of biotherapy of B-cell precursor leukemia by targeting genistein to CD19-associated tyrosine kinase.

Besides antiproliferation, plant isoflavonoids have a myriad of other putative effects, such as cholesterol reduction, antioxidant activity, and menopause symptom prevention. Our interest in isoflavonoids arises from the potential cardiovascular consequences of recent studies showing that the isoflavonoid phytoestrogen equol was (1) as potent as furosemide to inhibit Na-K-Cl cotransport fluxes in vitro in LLC-PK1 cells and human erythrocytes and (2) a natriuretic agent in the rat, although less potent than furosemide. This modest natriuretic potency of equol in vivo is probably due to its glucuro-conjugation and protein binding.

Vigne et al. reported that genistein inhibits Na-K-Cl cotransport in rat brain capillary endothelial cells, with IC50 of 35 μmol/L. Moreover, these authors found that the tyrosine kinase inhibitor herbimycin A (10 μmol/L) also reduced cotransport activity in rat brain endothelial cells. Interestingly, genistein was also shown to inhibit the Na-H antipporter in rabbit ileum and an amiloride-sensitive sodium conductance in A6 cells. In all cases, ion transport inhibition by genistein was related to its ability for inhibiting tyrosine kinase activity.

Here we confirmed in LLC-PK1 cells that genistein inhibits Na-K-Cl cotransport (IC50=34.7 μmol/L, Table 1). Whether this cotransport inhibitory action is due to tyrosine kinase inhibition was out of the scope of this study. Indeed, our present study was primarily designed to investigate if besides equol, other biologically relevant isoflavonoids possess natriuretic actions. In this respect, the IC50 value for cotransport inhibition by genistein in LLC-PK1 cells (34.7 μmol/L) was slightly higher than that previously reported for equol (23.6 μmol/L) in the same cell system. Conversely, daidzein was a poor cotransport inhibitor. Therefore, only genistein was investigated for salidiuretic actions in the rat.

The isolated, perfused rat kidney is a sensitive assay to investigate natriuretic substances. It retains almost completely the normal kidney tubular function and is isolated from compensatory mechanisms present in the whole animal. However, it is important to mention that there is some intersample variation in basal and stimulated salidiuresis that can explain, at least in part, the variability of the data (see Fig 2 through 4 and Tables 2 through 5). In IPK, genistein induced dose-dependent increases in diuresis, natriuresis, and kaliuresis. Maximal salidiuresis with genistein was quite similar as with furosemide. In terms of active dosis, genistein was 3 to 5 times less potent than furosemide and similar to equol.

Genistein failed to modify GFR but caused a statistically significant vasorelaxation of the isolated kidney, a preparation considered to have a poor vascular tone. Interestingly, this compound has already been shown to reverse the effects of several vasoconstrictors. On the other hand, indomethacin reduced renal vasodilatation by genistein, suggesting that this compound acts, at least in part, through the arachidonic acid cascade (for actions of flavonoids and isoflavonoids on the arachidonic acid cascade see Reference 18).

Several arguments suggested that genistein and furosemide share a common mechanism of salidiuretic action independent of changes in GFR. First, genistein and furosemide actions in isolated kidney function were not additive (although furosemide alone did not change RVR, it tended to reduce renal vasodilatation by genistein). Second, the inhibitor of prosta-

TABLE 2. Interactions of Genistein-Furosemide on IPK Salidiuresis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diuresis, μL/min per g Kidney</th>
<th>Natriuresis, μmol/min per g Kidney</th>
<th>Kaliuresis, μmol/min per g Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>15±2 (4)</td>
<td>0.66±0.17 (4)</td>
<td>0.24±0.07 (4)</td>
</tr>
<tr>
<td>Genistein, 308 μmol/L</td>
<td>74±14 (5)*</td>
<td>5.04±0.98 (5)*</td>
<td>0.85±0.11 (5)*</td>
</tr>
<tr>
<td>Furosemide, 100 μmol/L</td>
<td>49±3 (4)*</td>
<td>4.45±0.39 (4)*</td>
<td>0.85±0.08 (4)*</td>
</tr>
<tr>
<td>Genistein+furosemide</td>
<td>53±10 (4)* NS</td>
<td>4.98±1.14 (4)* NS</td>
<td>0.69±0.13 (4)* NS</td>
</tr>
</tbody>
</table>

*P<.05 comparing drug vs basal values; † P<.05 comparing genistein vs indomethacin; NS, not significant comparing genistein vs genistein+indomethacin.

IPK indicates isolated, perfused kidney.

The number of experiments is given in parentheses.

TABLE 3. Effect of Indomethacin on IPK Salidiuresis by Genistein

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diuresis, μL/min per g Kidney</th>
<th>Natriuresis, μmol/min per g Kidney</th>
<th>Kaliuresis, μmol/min per g Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>20±1 (4)</td>
<td>0.76±0.04 (4)</td>
<td>0.30±0.05 (4)</td>
</tr>
<tr>
<td>Genistein, 308 μmol/L</td>
<td>74±14 (5)*†</td>
<td>5.04±0.98 (5)*†</td>
<td>0.84±0.11 (5)*†</td>
</tr>
<tr>
<td>Indomethacin, 10 μmol/L</td>
<td>32±4 (4)</td>
<td>1.38±0.37 (4)</td>
<td>0.46±0.10 (4)</td>
</tr>
<tr>
<td>Genistein+indomethacin</td>
<td>53±5 (4) NS</td>
<td>3.49±0.58 (4) NS</td>
<td>0.70±0.14 (4) NS</td>
</tr>
</tbody>
</table>

*P<.05 comparing drug vs basal values; † P<.05 comparing genistein vs indomethacin; NS, not significant comparing genistein vs genistein+indomethacin.

IPK indicates isolated, perfused kidney.

The number of experiments is given in parentheses.
Glandin synthesis, indomethacin, reduced the salidiuretic action of genistein (for indomethacin inhibition of salidiuresis by furosemide see Reference 19). Finally, after perfusion of submaximal natriuretic doses of genistein, urinary concentrations of genistein reached the micromolar range (12 to 15 μmol/L), as expected for inhibition of Na-K-Cl cotransport (Table 1), note that the TALH “absorptive” Na-K-Cl cotransporter is a distinct protein isoform from the cotransporter expressed in other tissues20). It is important to mention that such urinary genistein concentrations are 1 to 2 orders of magnitude lower than those required to inhibit the Na-H antiporter in rabbit ileum14 or the amiloride-sensitive sodium conductance in A6 cells. (A6 cells are representative of the cortical segment of collecting tubules, where sodium reabsorption is small in the absence of aldosterone). Taken together, all these results suggest that salidiuresis by genistein results from inhibition of the TALH Na-K-Cl cotransporter.

Because of the low solubility of genistein, we chose the subcutaneous route for the in vivo experiments. Subcutaneous genistein induced significant increases in diuresis and natriuresis during the first 6 hours after administration. The same is observed with the so-called nondiuretic doses of furosemide, which increase salidiuresis in the first 6 hours but have no net effect when the complete period of 24 hours is considered21. The above results support the idea that inhibition of Na-K-Cl cotransporters by genistein and equol can offer new perspectives in isoflavonoid research. First, whether tyrosine kinases can be involved in cotransport regulation remains to be further investigated. Changes in cotransport activity were

### Table 4. Interactions of Genistein-Furosemide on IPK Hemodynamics

<table>
<thead>
<tr>
<th>Compound</th>
<th>GFR, μL/min per g Kidney</th>
<th>Relative RVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>387±21 (4)</td>
<td>0.98±0.01 (4)</td>
</tr>
<tr>
<td>Genistein, 308 μmol/L</td>
<td>383±31 (5)</td>
<td>0.84±0.02 (5)*</td>
</tr>
<tr>
<td>Furosemide, 100 μmol/L</td>
<td>399±35 (4)</td>
<td>0.95±0.02 (4)</td>
</tr>
<tr>
<td>Genistein+furosemide</td>
<td>337±31 (4)</td>
<td>0.94±0.02 (4)+ NS</td>
</tr>
</tbody>
</table>

IPK indicates isolated, perfused kidney; GFR, glomerular filtration rate; and RVR, renal vascular resistance (relative to basal values). *P<.05 comparing drug vs basal values; †P<.05 comparing genistein+furosemide vs genistein alone; NS, not significant comparing genistein+furosemide vs furosemide alone.

The number of experiments is given in parentheses.

### Table 5. Effect of Indomethacin on IPK Hemodynamic Changes by Genistein

<table>
<thead>
<tr>
<th>Compound</th>
<th>GFR, μL/min per g Kidney</th>
<th>Relative RVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>498±51 (4)</td>
<td>994±6 (4)</td>
</tr>
<tr>
<td>Genistein, 308 μmol/L</td>
<td>383±31 (5)</td>
<td>841±19 (5)*†</td>
</tr>
<tr>
<td>Indomethacin, 10 μmol/L</td>
<td>472±29 (4)</td>
<td>963±34 (4)</td>
</tr>
<tr>
<td>Genistein+indomethacin</td>
<td>326±62 (4)</td>
<td>928±37 (4) NS</td>
</tr>
</tbody>
</table>

IPK indicates isolated, perfused kidney; GFR, glomerular filtration rate; and RVR, renal vascular resistance (relative to basal values). *P<.05 comparing genistein vs basal values; †P<.05 comparing genistein vs indomethacin; NS, not significant comparing indomethacin vs genistein+indomethacin.

The number of experiments is given in parentheses.
associated to induction of differentiation of erythroleukemia cells\textsuperscript{22} and to inhibition of vascular endothelial cell proliferation.\textsuperscript{23} Indeed, cotransport participates in regulation of cell growth and cotransport inhibition by isoflavonoids could influence the in vitro effects of these substances on cell growth or proliferation, which were seen at similar concentrations.

Second, our results suggest that dietary genistein can influence renal function. In this respect, humans consuming soybean products can excrete micromolar concentrations of urinary genistein (6 to 15 \mu mol/L, Reference 24). These values compare well with those required to produce salidiuresis and decrease vascular resistance in the isolated, perfused rat kidney (12 to 15 \mu mol/L of urinary genistein for submaximal natriuretic action). However, whether dietary genistein can be natriuretic in humans consuming soybean products remain as an open question because a large fraction of urinary genistein is glucuron conjugated and sulfoconjugated.\textsuperscript{25} Moreover, (1) other isoflavonoids are present in vegetarian diet and can perhaps be also natriuretic and (2) the sulfoconjugated metabolites of genistein are perhaps natriuretic in humans (for natriuretic actions of sulfoconjugated metabolites see Reference 26). Therefore it seems interesting to investigate if natriuretic actions could be reached with dietary supplements or administration of purified isoflavonoids proposed for other healthy effects or by a vegetarian diet containing isoflavonoids.

Regarding the therapeutic potential of genistein as a new natriuretic drug, its salidiuretic profile has no significant advantage with respect to that of furosemide. Moreover, orally given genistein might have generalized side effects due to the general importance of tyrosine kinases in cellular function. Therefore we do not believe that genistein can be reasonably developed as a new diuretic drug.

The potential importance of natriuretic actions of dietary isoflavonoids arises from the several reports showing low blood pressure in vegetarians (ie, References 27 and 28). In this respect, controlled intervention studies argue against a role in hypertension for animal products or their associated saturated fats.\textsuperscript{29} Furosemide compares well with furosemide in the isolated, perfused rat kidney.\textsuperscript{23} Indeed, cotransport participates in regulation of cell growth and cotransport inhibition by isoflavonoids could influence the in vitro effects of these substances on cell growth or proliferation, which were seen at similar concentrations.

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In conclusion, genistein possesses a similar salidiuretic profile and compares well with furosemide in the isolated, perfused rat kidney and is also natriuretic by the subcutaneous route in the rat. Further investigation is required to see if dietary genistein can be natriuretic through the inhibition of the TALH Na–K–Cl cotransporter.

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